

## Report

# Activation of the Endosome-Associated Ubiquitin Isopeptidase AMSH by STAM, a Component of the Multivesicular Body-Sorting Machinery

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## Summary

AMSH is an endosomal ubiquitin isopeptidase that can limit EGF receptor downregulation[1]. It directly binds to the SH3 domain of STAM, which is constitutively associated with Hrs, a component of clathrin-coated structures on endosomes. This clathrin coat has been implicated in the recruitment of ubiquitinated growth factor receptors prior to their incorporation into internal vesicles of the multivesicular body (MVB) [2, 3], through the concerted action of ESCRT complexes I, II, and III [4]. We now show that AMSH is embedded within a network of interactions with components of the MVB-sorting machinery. AMSH and STAM, like Hrs [5], both bind directly to clathrin. AMSH also interacts with mVps24/CHMP3, a component of ESCRT III complex, and this interaction is reinforced through simultaneous STAM binding. We have explored the effect of interacting components on the *in vitro* enzymatic activity of AMSH. The enzyme shows specificity for K63- over K48-linked polyubiquitin chains *in vitro* and is markedly stimulated by coincubation with STAM, indicating that activation of AMSH is coupled to its association with the MVB-sorting machinery. Other interacting factors do not directly stimulate AMSH but may serve to orient the enzyme with respect to substrates on the endosomal membrane.

## Results and Discussion

Internalized receptors are delivered to a tubulo-vesicular compartment known as the early or sorting endosome. From here, receptors may recycle to the plasma membrane or be selected for lysosomal sorting by incorporation into small vesicles that bud away from the limiting membrane into the vacuolar lumen to generate multivesicular bodies (MVBs). Ubiquitination of tyrosine kinase receptors provides a sorting signal that interacts with several components of the MVB-sorting machinery [6]. Deubiquitinating enzymes (DUBs) may function at the endosome to oppose E3-ligase activity or to recycle ubiquitin from receptors that have been committed to

the sorting pathway. AMSH is an endosomal DUB belonging to the JAMM metalloprotease family [1]. siRNA-mediated knockdown of AMSH leads to increased rates of EGFR degradation, consistent with enhanced targeting to the lysosomal pathway [1].

AMSH interacts with the SH3 domain of STAM, a component of the MVB pathway that is constitutively associated with Hrs. The Hrs-STAM complex provides the first point of engagement of ubiquitinated receptors with the sorting machinery and, through Hrs binding to clathrin, may serve to concentrate receptors within specialized endosomal clathrin coats. We introduced point mutations within the STAM binding motif PXV/ID/NRXXKP [7] of AMSH (RKP) that abolish association with STAM, and we reciprocally deleted the STAM SH3 domain ( $\Delta$ SH3) to abrogate binding to AMSH (Figure 1A). We then analyzed the effect of STAM coexpression on the subcellular distribution of AMSH. GFP-AMSH shows a nuclear and cytosolic localization that includes endosomes, which we find more prominently labeled by catalytically inactive mutant (D348A) [1]. When coexpressed with HA-STAM, we observed a dramatic recruitment of GFP-AMSH (D348A) to endosomes, effectively draining the nuclear pool (Figure 1B, d–f). This depends upon AMSH binding to STAM, as it is abolished by introducing a mutation in the STAM binding motif (RKP) (Figure 1B, g–i) or by deleting the SH3 domain in STAM (not shown). STAM-dependent redistribution of AMSH from nucleus to endosomes is only captured by catalytically inactive enzyme, perhaps because the interaction is normally transient and the AMSH catalytic cycle is linked to its dissociation from the membrane. *In vitro* experiments indicate equal binding affinities of STAM to catalytically active and inactive forms of AMSH (Figure 1C). STAM containing a mutation in its UIM domain, which can neither bind to ubiquitin nor be ubiquitinated, no longer elicits AMSH (D348A) redistribution, while its interaction with AMSH is unimpaired (Figures 1C and 1D). It is possible that ubiquitin, which accumulates on endosomes after GFP-AMSH(D348A) expression [1], cooperates with STAM to retain AMSH on the endosomal membrane. Interestingly, expression of catalytically inactive AMSH leads to the accumulation of a ubiquitinated form of STAM itself [1]. This effect requires direct interaction with STAM as the combination of STAM binding (RKP), and catalytically inactivating mutations does not lead to a corresponding accumulation (see Figure S3 in the Supplemental Data available with this article online).

Immunoprecipitation of GFP-AMSH or a catalytically inactive mutant GFP-AMSH(D348A) from transiently transfected HEK293T cells revealed a specific protein band copurifying with AMSH (Figure 2A). We identified this as clathrin heavy chain by peptide mass fingerprinting and subsequent Western blotting (Figure 2B and Figure S1). Pull-down experiments with purified proteins demonstrated a direct interaction between AMSH and the clathrin-terminal domain, which mediates many of

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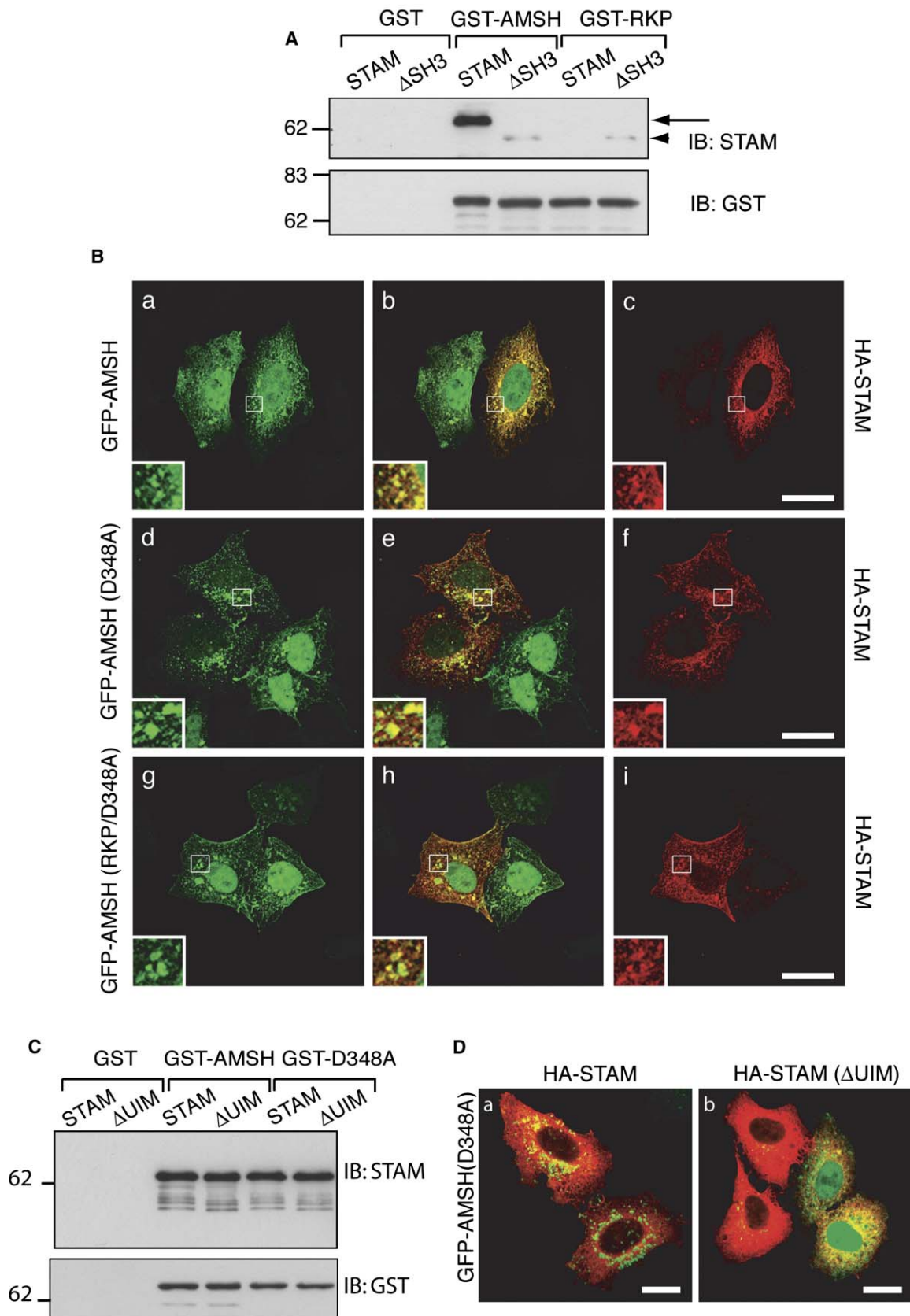
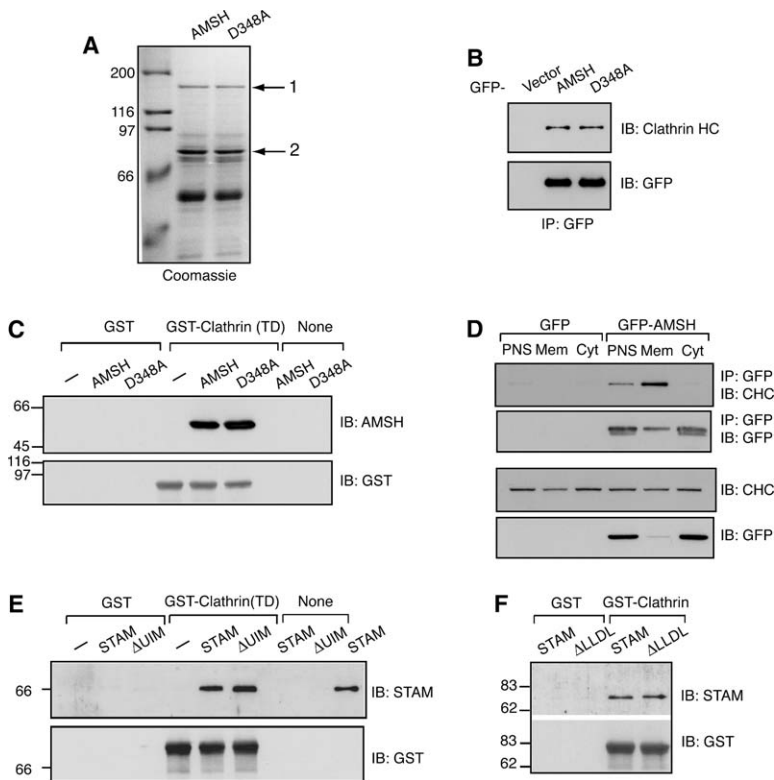


Figure 1. STAM Redistribution of AMSH to Endosomes Requires Direct Association and an Intact UIM Domain

(A) Purified His<sub>6</sub>-STAM or His<sub>6</sub>-STAM-SH3 deletion mutant ( $\Delta$ SH3) were incubated with GST, GST-AMSH, or a STAM binding mutant, GST-AMSH-RKP, and isolated with glutathione sepharose beads.



**Figure 2. Identification of Clathrin as a Novel Binding Partner of AMSH and STAM**

(A) HEK293T cells were transfected with GFP-AMSH or GFP-AMSH (D348A), and GFP-tagged proteins were immunoprecipitated with anti-GFP. Bound proteins were resolved by SDS-PAGE and stained with Coomassie blue. The first lane shows molecular weight markers. Bands indicated by arrows were analyzed by MALDI-TOF and identified by peptide mass fingerprinting as (1) clathrin (see Figure S1) and (2) GFP and AMSH.

(B) Immunoprecipitates were obtained as in (A), then immunoblotted with anti-clathrin heavy chain (HC) or anti-GFP.

(C) The terminal domain of clathrin heavy chain interacts directly with AMSH. AMSH or catalytically inactive D348A-AMSH were incubated by themselves (none), with GST or GST-clathrin heavy chain terminal domain (TD). GST-tagged proteins were isolated with glutathione sepharose beads and analyzed for AMSH binding by immunoblotting with anti-AMSH and anti-GST.

(D) AMSH and clathrin interact exclusively on membranes. HeLa cells were transfected with GFP or GFP-AMSH and homogenized. Postnuclear supernatant (PNS) as well as cytosolic (Cyt) and membrane (Mem) fractions were prepared from HeLa cells transfected with GFP or GFP-AMSH. After immunoprecipitation with anti-GFP, bound proteins were analyzed by immunoblotting with anti-Clathrin (CHC) or anti-GFP. 30  $\mu$ g of PNS,

the equivalent volume of cytosol, as well as 3-fold the corresponding amount of membrane fractions were also loaded.

(E) STAM interacts directly with the clathrin heavy chain terminal domain. STAM or a UIM point mutant of STAM ( $\Delta$ UIM) were incubated by themselves (none), with GST, or with GST-clathrin heavy chain terminal domain (TD). Bound proteins were isolated as in (C) and analyzed by immunoblotting with anti-STAM and anti-GST.

(F) Purified His-STAM (22 pmoles) or a canonical clathrin binding motif mutant of STAM ( $\Delta$ LLDL) (22 pmoles) were incubated with GST or GST-clathrin terminal domain (both 55 pmoles) and analyzed as in (E).

the known interactions with clathrin (Figure 2C). There are large cytosolic pools of both these proteins in cells; however, their interaction is exclusive to membrane fractions (Figure 2D).

As STAM possesses a canonical clathrin binding motif (LLDLE), we also examined its clathrin binding properties. In common with several other clathrin-interacting proteins, STAM specifically binds to the terminal domain of clathrin, although this interaction is insensitive to mutation of the predicted binding site (Figures 2E and 2F). Thus, the STAM-Hrs-AMSH complex may engage in a tripartite interaction with clathrin, embedding it within the endosomal coat structure.

Hrs serves to recruit downstream components of the MVB-sorting machinery, most notably the ESCRT I complex through interaction with TSG101 [8]. This in turn is believed to trigger recruitment of ESCRT complexes II and III that are necessary for vesicle formation [4]. While the initial functional genetic studies in yeast suggested a simple vectorial recruitment and association of these

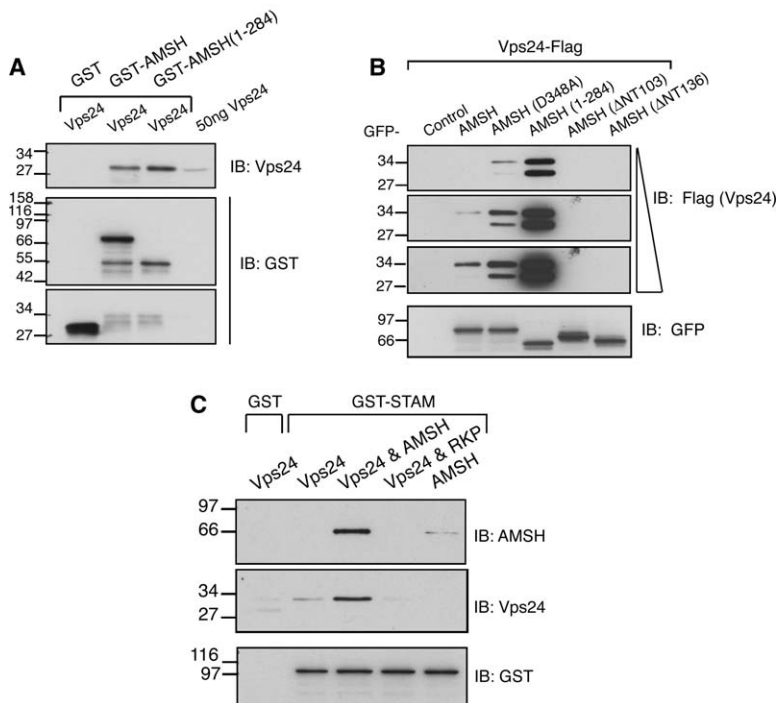
three complexes, further directed yeast two-hybrid interaction data indicate a much more complex interaction network [9, 10]. A genome-wide two-hybrid screen suggests that the *Drosophila* homolog of AMSH may interact with components of the ESCRT III complex, Vps24/CHMP3 and Snf7/CHMP4 [11], that are involved in the final stages of luminal vesicle formation. We tested the interaction of mammalian Vps24 (mVps24) with AMSH and with a C-terminal truncated form, AMSH (1-284). Both interact directly with His<sub>6</sub>-mVps24 (Figure 3A). Coexpression of Vps24-Flag with various GFP-tagged AMSH constructs in HEK293T cells recapitulates the in vitro interaction and maps the interaction site to the N-terminal domain of mVps24 (Figure 3B).

We wondered whether AMSH can interact simultaneously with STAM and mVps24. Therefore, we asked if STAM can pull down mVps24 when coincubated with AMSH, through the direct association between AMSH and STAM. However, our control incubation of STAM with mVps24 indicated a weak direct interaction

(B) HeLa cells were transfected with GFP-AMSH (a–c), GFP-AMSH (D348A) (d–f), or GFP-AMSH (RKP/D348A) (g–i) together with HA-STAM and stained with anti-HA (red). All panels show a single confocal section. Insets show 3-fold magnification of the boxed area. Scale bars equal 20  $\mu$ m.

(C) His<sub>6</sub>-STAM or a His<sub>6</sub>-STAM UIM-domain mutant ( $\Delta$ UIM: L176A, S177A) were incubated with GST, GST-AMSH, or GST-AMSH(D348A) and isolated as in (A).

(D) HeLa cells were transfected with GFP-AMSH (D348A) together with HA-STAM or HA-STAM ( $\Delta$ UIM) and stained as in (B). Scale bars equal 20  $\mu$ m.



**Figure 3. Vps24 Interacts Directly and Simultaneously with AMSH and STAM**

(A) Purified His<sub>6</sub>-Vps24 was incubated with GST, GST-AMSH, or a C-terminal AMSH deletion mutant, GST-AMSH (1-284), at 4°C for 1 hr. The proteins were isolated with glutathione sepharose beads, followed by immunoblotting with anti-Vps24 and reprobing with HRP-coupled anti-GST.

(B) Lysates prepared from HEK293T cells co-transfected with Vps24-Flag and GFP-tagged AMSH, catalytically inactive AMSH (D348A), AMSH (1-284), or N-terminal deletion mutants AMSH (ΔNT103) and AMSH (ΔNT136) were subjected to immunoprecipitation with anti-GFP. Associated Vps24 was detected by immunoblotting with HRP-coupled anti-Flag. To allow visualization of all bands, three different exposures of the blot are shown.

(C) His<sub>6</sub>-Vps24 (22 pmol) was incubated with GST or GST-STAM (22 pmol) in the absence or presence of AMSH or AMSH (RKP) (22 pmol) at 4°C for 1 hr isolated as in (A) and probed with anti-Vps24 or anti-AMSH antibodies and subsequently reprobed with HRP-coupled anti-GST.

between these two proteins (Figure 3C). Importantly, this interaction is strongly enhanced by coincubation with AMSH, and reciprocally AMSH interaction with STAM is reinforced by mVps24, indicating that the complex is stabilized through a set of mutual interactions. This effect is lost when the STAM binding motif in AMSH is mutated (RKP). Hence, AMSH can simultaneously interact with both mVps24 and STAM, providing a direct connection between Hrs-STAM and ESCRTIII complexes. Overall, our interaction data reveal AMSH to be more deeply integrated as a hub protein within the MVB-sorting protein interaction network than hitherto appreciated.

AMSH is so far unique among JAMM domain-containing proteins in displaying measurable isopeptidase activity in isolation. Other JAMM family proteins such as Rpn11 and Csn5 must be embedded in large multi-protein complexes of the proteasome and COP9 signalosome, respectively, for any processing activity to be evident [12–14]. We wondered whether AMSH engagement with endosomal partner proteins could likewise regulate its activity.

In vitro DUB assays with enzymatically produced tetra-ubiquitin chains demonstrate strong selectivity of AMSH for K63-linked versus K48-linked chains (Figure 4A). The significance of this specificity may relate to roles for K63 ubiquitin in both endocytic sorting and signal transduction cascades that have been proposed [15, 16]. There is some evidence that certain endosomal cargo proteins in yeast are modified with K63-linked di-ubiquitin [16]. This may simply provide for higher affinity interactions with ubiquitin binding domains than mono-ubiquitin, while still avoiding proteasomal degradation. AMSH can also deubiquitinate receptors such as EGFR [1] and PDGFR (our unpublished observations), which have been proposed to be multimonoubiquitinated [17, 18]. K63-linked polyubiquitin chains adopt a more

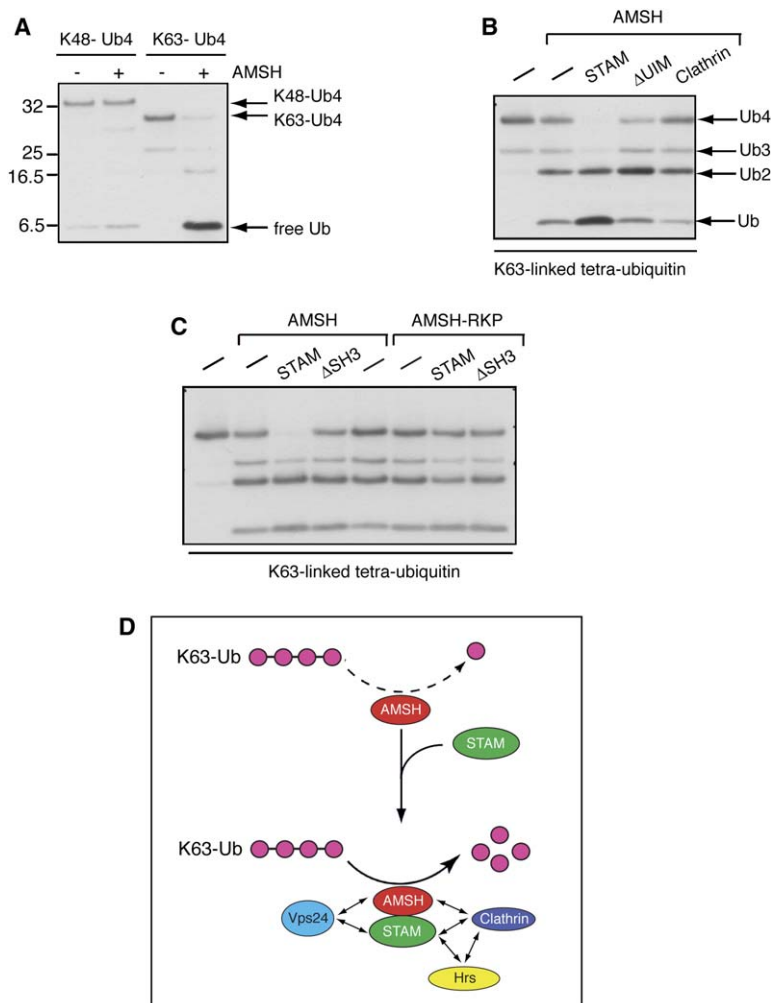
open configuration than K48-linked ubiquitin and may more closely resemble monoubiquitin linked to substrate proteins [19].

Hrs, clathrin-TD, full-length clathrin, or mVps24 had no effect on AMSH activity (Figure 4B, Figure S2). In contrast, coincubation with STAM markedly increased the rate of AMSH-dependent processing of K63-linked tetra-ubiquitin (Ub<sub>4</sub>) (Figure 4B), while STAM displays no independent DUB activity (Figure S2A). Stimulatory activity requires direct interaction as it is abolished by deletion of the STAM SH3 domain and by the corresponding mutations in the STAM binding motif in AMSH (RKP) (Figure 4C). It is also greatly reduced by point mutations in the UIM domain of STAM (Figure 4B), which do not interfere with binding to AMSH (Figure 1C). Our DUB assay is not currently amenable to testing if STAM is enhancing  $k_{cat}$  or  $K_M$ ; however, the requirement for an intact UIM domain within STAM may suggest the latter. By providing an accessory ubiquitin binding site, this domain could increase the affinity of the AMSH-STAM complex for substrate. We find that STAM also stimulates in vitro AMSH DUB activity toward EGFR immunoprecipitated from EGF-stimulated cells, likewise dependent upon direct interaction between AMSH and STAM (Figure S2E).

There is an approximate 10-fold excess of proposed ubiquitin E3-ligases (~1000) over DUBs (~100) in the human genome [20, 21]. One could infer that DUBs have broader substrate specificity in vivo. However, many E3s (most likely all RINGs) are simply adaptor proteins for E2 ligases of which there are about 50 in the genome. Specificity is the product of the E2/E3 combination. We propose that STAM fulfills an adaptor function for AMSH and that there might be a correspondingly diverse set of DUB/adaptor combinations.

The ability of STAM to directly stimulate AMSH activity serves to couple localization and enzyme activity, illustrating an important principle of cellular enzymology.





**Figure 4. STAM Stimulates the Activity of the K63-Specific Ubiquitin Isopeptidase, AMSH**  
(A) 250 ng of K48- or K63-linked tetraubiquitin chains (K48-Ub<sub>4</sub>, K63-Ub<sub>4</sub>) were incubated with AMSH (1 μM) for 2 hr at 37°C as indicated. Specific processing of K63-Ub<sub>4</sub> to free ubiquitin can be observed.  
(B) AMSH (1 μM) was preincubated at 4°C for 30 min with STAM (5 μM), STAM-ΔUIM (5 μM), or clathrin terminal domain (5 μM) and then added to 250 ng K63-Ub<sub>4</sub> for 15 min at 37°C.  
(C) AMSH (0.5 μM) or a STAM binding mutant of AMSH (AMSH-RKP) (0.5 μM) were preincubated with STAM or STAM (ΔSH3) (1 μM) and then added to K63-Ub<sub>4</sub> for 15 min at 37°C.  
(D) STAM activates AMSH DUB activity toward K63-linked ubiquitin chains. The AMSH-STAM complex engages in a network of interactions, which may serve to stabilize the complex and contribute to substrate recognition.

Other components of the AMSH interaction network (Hrs, clathrin, Vps24) do not influence AMSH activity *in vitro* (Figure S2). However, our *in vitro* assay, wherein components have three translational degrees of freedom, may fail to encapsulate details of a reaction confined to the two-dimensional surface of endosomes. In this situation, interacting partners may serve to orientate the enzyme with respect to the clathrin coat and membrane surface for optimal expression of AMSH function (Figure 4D).

#### Supplemental Data

Supplemental Data include three figures and Supplemental Experimental Procedures and can be found with this article online at <http://www.current-biology.com/cgi/content/full/16/2/160/DC1/>.

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